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Differentiation of Influenza B Virus Lineages Yamagata and Victoria by Real-Time PCR[▽]

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Since the 1970s, influenza B viruses have diverged into two antigenically distinct virus lineages called the Yamagata and Victoria lineages. We present the first real-time PCR assay for virus lineage differentiation to supplement classical antigenic analyses. The assay was successfully applied to 310 primary samples collected in Germany from 2007 to 2009.

Influenza viruses are members of the family *Orthomyxoviridae* and are divided into three genera, A, B, and C (8). Influenza A and B viruses are most relevant clinically, since they cause severe respiratory infections in humans (2). While influenza A viruses comprise a large group of different subtypes (8), influenza B viruses formed a homogenous group and started to diverge into two antigenically distinguishable lineages only in the 1970s (3, 4, 6). These virus lineages were named after their first representatives, B/Victoria/2/87 and B/Yamagata/16/88, as the Victoria and Yamagata lineages (6). Today, the antigenic differences between the lineages allow their differentiation by hemagglutination inhibition testing (HIT) by using specific immune sera raised against contemporary strains of either lineage. However, HIT is a time-consuming and tedious process and needs virus isolation as a prerequisite. In contrast, PCR is well known to be a fast, specific, and sensitive diagnostic method, and furthermore, real-time PCR reduces the risk of carryover contamination and allows large-scale diagnostics (5). However, to date, there has been no real-time PCR assay described that enables the differentiation of influenza B viruses, which would greatly speed up and thus improve influenza virus surveillance. We therefore present an assay that not only amplifies viruses of both lineages but also discriminates between them by the application of two differently labeled minor-groove binder (MGB) probes, with either one being specific for one lineage.

The target region of the assay was chosen from an alignment with recent influenza B virus hemagglutinin (HA) database sequences (from the years 2000 to 2008). The 81-bp amplicon comprises a 13-bp stretch that differs in 6 positions between the two lineages. The stability of the characteristic nucleotide changes was confirmed by an alignment comprising all available influenza B virus hemagglutinin database sequences (1,622 sequences, from the years 1954 to 2008). The distinctive nucleotides have been stable from the late 1990s until today, so nucleotide changes are not impossible but are unlikely to occur in the near future. Thus, an MGB probe was designed for either lineage targeting this 13-bp stretch. By the application of both probes with different color labels (6-carboxyfluorescein [FAM] and VIC) in a single PCR, both virus lineages can be detected and discriminated simultaneously, as only one of the two probes will give a fluorescence signal.

Reaction conditions were established for the LightCycler 480 system in a total reaction mixture volume of 25 μl containing 1× PCR buffer, 5 mM $MgCl_2$, 1.25 μM deoxynucleoside triphosphate (dNTP) (Invitrogen) with dUTP (GE Healthcare, Great Britain), 0.5 U Platinum *Taq* polymerase (Invitrogen), 900 nM forward primer F432 (5′-ACCCTACARAMTTGGAACYTCAGG-3′), 600 nM reverse primer R479 (5′-ACAGCCCAAGCCATTGTTG-3′), 150 nM Yamagata probe MGB437 (5′-FAM–AATCCGMTYTTACTGGTAG–MGB-3′), 100 nM Victoria probe MGB470 (5′-VIC–ATCCGTTTCCATTGGTAA–MGB-3′), and 3 μl of template cDNA. Cycling conditions were 5 min at 95°C, followed by 45 cycles of 15 s at 95°C and 30 s at 60°C.

The assay was evaluated by using two plasmids that were cloned according to routine procedures (1) and contained 610 and 613 bp of the hemagglutinin genes of B/Bayern/7/08 (plasmid pYam) and B/Berlin/38/08 (plasmid pVic), two contemporary German isolates representing the Yamagata and Victoria lineages, respectively. Thus, the complete primer- and probe-binding regions represent the original sequences of these two isolates. Amplification of 10-fold serial dilutions of each plasmid in λ DNA (1 ng/μl) revealed a linear detection range from 10^7 to 10^2 genome equivalents per reaction with a correlation (R^2) of >0.998 and slopes of −3.32 (pYam) and −3.33 (pVic) (Fig. 1A), resembling a PCR efficiency of 1 ($E = 10^{-1/\text{slope}} - 1$). We performed a probit analysis as a model of nonlinear regression that indicated a 95% detection probability of 24.4 genome equivalents per reaction for plasmid pYam and 12.4 genome equivalents per reaction for pVic (Fig. 1B). Additionally, from virus culture material of the corresponding virus isolates B/Bayern/7/08 (Yamagata) and B/Berlin/38/08 (Victoria), the 95% detection probabilities were determined to be 1.3×10^{-5} and 3.8×10^{-5} HA units per reaction, respectively. The overall variability was assessed by the repeated examination of three different plasmid copy numbers as well as virus culture material with a high, medium, or low virus load. The standard deviations of threshold cycle (C_T) values were found to be very low and were comparable for Yamagata and Victoria viruses and plasmids (Table 1). We found no cross-

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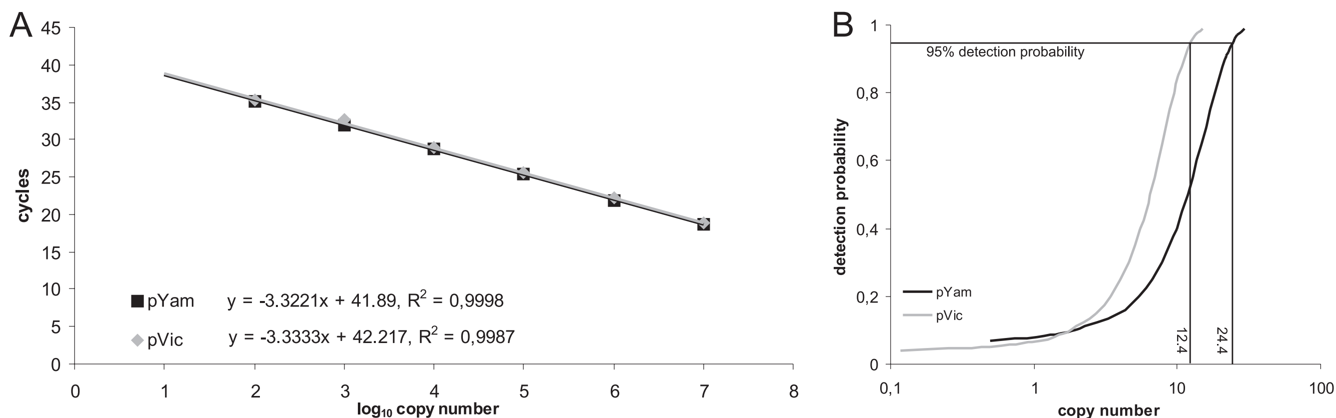

FIG. 1. PCR assay validation. (A) Mean C_T values (double reactions) of plasmid dilutions containing 10^7 to 10^2 genome equivalents of pYam and pVic were plotted against the cycle number. The slope and correlation (R^2) are indicated. (B) Probit analyses were performed by examination of plasmid dilutions containing 100 to 0.1 genome equivalents of pYam and pVic in 10-fold reactions. Results were analyzed by using SPSS 17.0 statistics software.

reactivity with DNA/cDNA of isolates from seasonal influenza A virus subtypes H1N1 and H3N2; pandemic influenza A/H1N1 virus; respiratory syncytial viruses A and B; adenovirus serotypes 2, 3, and 4; human metapneumovirus; parainfluenza viruses 1, 2, and 3; coxsackievirus; and rhinovirus as well as human DNA from swab samples.

Finally, to confirm the applicability of the assay to clinical diagnostics, we examined 310 influenza B virus-positive primary samples from the 2007-2008 and 2008-2009 influenza seasons. All samples were taken from German patients presenting with influenza-like illness and successfully underwent HIT after virus isolation on MDCK2 cells. The nasal and throat swabs were washed in minimal essential medium (MEM) cell culture medium immediately after arrival. RNA was extracted by using either the RTP DNA/RNA virus MiniKit (Invitek) or the MagAttract viral RNA M48 kit (Qiagen) according to the manufacturer's suggestions. cDNA was synthesized from 25 μl of RNA by applying Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) and random hexamer primers as described elsewhere previously (7). Residual RNA was stored at −80°C until further use.

TABLE 1. PCR assay validation: detection variability[a]

Variability	Material	Virus load (no. of genome equivalents/reaction)	SD of C_T value	
			Yamagata	Victoria
Intra-assay	Plasmids	5×10^5	0.03	0.05
		5×10^3	0.12	0.08
		5×10^1	0.34	0.54
	Cultured virus	High	0.08	0.16
		Medium	0.21	0.10
		Low	0.22	0.35
Interassay	Plasmids	5×10^5	0.04	0.07
		5×10^3	0.17	0.11
		5×10^1	0.64	0.56
	Cultured virus	High	0.35	0.34
		Medium	0.27	0.40
		Low	0.23	0.39

[a] Variability runs were performed by examination of pYam and pVic plasmid dilutions (5×10^5, 5×10^3, and 5×10^1 genome equivalents per reaction) as well as cultured virus material with a high (6.67×10^8 genome copies/ml), medium (6.67×10^6 genome copies/ml), or low (6.67×10^4 genome copies/ml) virus load. Intra-assay variability was tested in sextuplicate reactions. Interassay variability was determined by 2-fold examinations of duplicate reactions with the inclusion of data from the intra-assay variability run (total, 3-fold examination). The standard deviations (SD) of obtained C_T values are listed.

By applying the presented assay, viruses were amplified from all 310 primary samples with C_T values between 22 and 37. All samples were genetically identified as Yamagata or Victoria lineage viruses in concordance with HIT results. The 310 primary samples comprised 185 Yamagata and 3 Victoria lineage viruses from the 2007-2008 season as well as 120 Victoria and 2 Yamagata lineage viruses from the 2008-2009 season. Since the assay's introduction into our diagnostic routine in February 2009, it has been run on approximately 5,000 samples, and to our knowledge, no false-positive or false-negative results have been obtained.

In summary, we present the first real-time PCR assay for the differentiation of influenza B viruses. This assay speeds up virus lineage identification in clinical specimens considerably and will therefore help to improve the surveillance of influenza B viruses. Furthermore, it will enable a timely recognition of the circulating B virus lineage during influenza seasons and will thus allow short-term decisions on patient care, e.g., in the case of a nonmatching vaccine, as well as the early onset of on-time epidemiological examinations, including WHO decisions on vaccine composition.

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